Amendments to the Specification

At the indicated page and line numbers, please replace the existing paragraphs with the ones set forth below.

(Page 12, lines 4 through 19)

FIGURE 6. In vitro chemical ligation of folded recombinant proteins is illustrated by the separation of Abl-SH(32). The Abl-SH3 domain is generated as an $^{\alpha}$thioester-derivative, and the Abl-SH2 domain is generated with a Cys at the N-terminus. The former is achieved using a modified version of expressed protein fixation (60, 61). Combining the two proteins under conditions which maintain them as folded results in a chemoselective ligation reaction and the generation of a normal peptide bond at the ligation junction (69). The sequence of the final ligation product is m{65}LFVALYDFVAS GDNTLSITKGEKLRVLGYNHNGEWAEAQTKNGQGWVPSNYITPVGCLEKHSWYHGPVSRNAA EYLLSSGINGSFLVRESESSPGQRSISLRYEGRVYHYRINTASDGKLYVSSESRFNTLAELV HHHSTVADGLITTLHYPAPKR{220}gihrd (SEQ ID NO: 1). Lower case letter indicate non-gene residues from the expression systems used. This construct uses a $C^{101}S$ mutation internal to the SH-3 which had previously been inserted to improve stability for NMR experiments. This is also in the `wildtype' sequence. Native chemical ligation reactions can be performed in the presence of multiple internal cysteine residues in either of the reacting segments (84); only the N-terminal cysteine participates in the ligation reaction.

(Page 23, line 21 through page 24, line 3)

The feasibility of the semi-synthetic approach of the present invention was first explored in a series of model studies. As a test system, a short recombinant fragment corresponding to amino acids 500-567 of the 613 amino acid-long *E. coli* RNA polymerase σ^{70} subunit was genetically fused to the intein-CBD, overexpressed and purified by affinity chromatography on chitin beads. Exposure of immobilized intein-fusion constructs to free cysteine has been shown to induce cleavage (38), and

indeed this was confirmed in the present system. It was then evaluated whether the immobilized construct could be chemically ligated to a short synthetic peptide (NH_2-Cys-Glu-Asp-Asn-Glu-Tyr-Thr-Ala-Arg-Glu-aminocaproate-Lys-ϵ-[fluoroscein]-CO_2H; SEQ ID NO: 2) containing an N-terminal cysteine to facilitate ligation and a C-terminal fluorescein reporter group. Initial studies in which the beads were simply treated with a solution containing 1 mM peptide at pH 7.3 were unsuccessful and neither ligation nor protein cleavage was detected, even after prolonged incubations.

(Page 35, lines 10 through 25)

With these results, the tyrosine phosphorylated and unphosphorylated forms of the peptide NH_2-Cys-Glu-Asp-Asn-Glu-Tyr-Thr-Ala-Arg-Glu-aminocaproate-Lys-ϵ-[fluorescein]-CO_2H (SEQ ID NO: 2) were designed and synthesized. Phosphorylated and unphosphorylated peptides were manually synthesized by Boc and Fmoc solid phase peptide synthesis, respectively. Phosphotyrosine was introduced during Fmoc chain assembly in the phosphate unprotected form. Orthogonal protection of the $-NH_2$ group of the C-terminal Lys residue with either Fmoc (Boc-strategy) or dde (Fmoc strategy) allowed direct attachment of fluorescein (activated as an Nhs ester) prior to the final cleavage step. Following cleavage, peptides were purified to homogeneity by HPLC and characterized by electrospray mass spectrometry. This sequence is derived from the highly conserved activating autophosphorylation site of Src family kinases (9, 10). In its tyrosine phosphorylated form, a similar sequence has been shown to bind specifically to the SH2 domain of Csk (21-23). The N-terminal cysteine residue was included in the peptide to facilitate native chemical ligation. Incorporation of a carboxy-terminal fluorescent tag via a flexible linker was envisaged to serve as a sensitive marker of successful ligation and as a probe for further biochemical studies.

(Page 40, lines 1 through 9)

All peptides were chemically synthesized according to optimized Boc SPPS (15), and purified by preparative reverse-phase HPLC using a Vydac C-18 column. In all cases, peptide composition and purity was confirmed by electrospray mass spectrometry and analytical HPLC. Fluoroscein was attached to the ϵ-amino group of the lysine residue in the peptide NH_2-CEDNEYTARE-aminocaproate-K-CO_2H (SEQ ID NO: 3) prior to the final cleavage/deprotection step using a Boc-Lys-ϵ-(NH-Fmoc) orthogonal protection strategy. The construct His_6-Cys-[SCH_2]-aminocaproate-$\sigma_{568-600}$ was prepared by chemically ligating the purified, unprotected peptides NH_2-His_6Cys-CO_2H and BrAc-aminocaproate-$\sigma_{568-600}$ using the previously described thioether-based chemical ligation strategy (10).

(Page 43, lines 12 through 27)
Initial attempts to generate the [C^{121}]SH2 construct involved cyanogen bromide cleavage of a GST-Abl-SH(32) fusion containing a unique Met-Cys unit at the appropriate position within the intein-domain linker. The Met-Cys unit was introduced into the linker region connecting the Abl-SH3 Zinc Abl-SH2 domains by cassette mutagenenesis using, a NcoI and XmaI restriction strategy. This resulted in Asn 120-→Met and Ser 121 Cys mutations in the Abl-SH(32) construct. The Abl-SH(32) sequence does not contain any endogenous Met residues. This synthetic strategy was unsuccessful because of irreversible oxidation of the cysteine residue to cysteic acid during the chemical cleavage step; the resulting CYS(O_3H)-Abl-SH2 analog could not participate in subsequent chemical ligation reactions. An alternative approach was therefore employed which utilized the Factor Xa cleavage strategy previously described by Verdine and co-workers (71). In this approach a GST-Abl-SH(32) fusion protein was generated which contained an -Ile-Glu-Gly-Arg-Cys- (SEQ ID NO: 9) motif within the linker region connecting the Abl-SH3 and Abl-SH2 domains. Proteolysis of this fusion protein with Factor Xa afforded the desired [C^{121}]SH2 construct in good yield. A

similar strategy was also used to prepare uniformly ^{15}N labeled [C^{121}]SH2 (see Materials and Methods).

(Page 43, line 29 through page 44, line 5)
Preliminary ligation studies investigated whether a short synthetic peptide, NH_2-CGRGRGRK[fluorescein]-$CONH_2$ (SEQ ID NO: 4) could be reacted with the immobilized Abl-SH3-intein-CBD fusion protein. Consistent with previously published examples (60, 61) nearly quantitative ligation of the synthetic peptide to the recombinant Abl-SH3 domain was observed, as indicated by reverse-phase HPLC, ESMS and fluorescence spectroscopy. These studies thus established that expressed protein ligation reactions could be performed on the folded Abl-SH3 domain.

(Page 45, line 21 through page 46, line 2)
Preliminary studies had indicated that HPLC purified recombinant Abl-SH(32) could be lyophilized and then refolded by rapid dilution from a 6 M GLIHCI containing buffer into phosphate buffer at pH 7.2. Under these conditions, no protein precipitation was observed and NMR analysis indicated the sample had adopted a native fold. A similar strategy was therefore used to prepare the complete [SH2-^{15}N]SH(32) construct for functional and structural analysis. The binding affinity of Abl-[SH2^{15}N]SH(32) for the consolidated ligand, NH_2-PVpYENVG_6>(PPAYPPPPVPK$_{CONH2}$) (SEQ ID NOs: 5 and 6, respectively), which binds both the SH3 and the SH2 domains simultaneously (78) was studied using a fluorescence-based titration assay. (The C-terminal glycyl residue is linked to the N.sub..epsilon. of lysyl in the second peptide segment). This revealed the equilibration dissociation constant (Kd) for binding to the ligand, 300 nM, was essentially that previously reported for the Abl-SH(32) construct, 249 nM (78). This affinity is characteristic of the dual domain construct.

(Page 47, line 18 through page 48, line 18)
Cloning and Expression of Abl [C^{121}]SH2: Suitable SH2 constructs were generated from a pGEX2T vector containing the

human Abl-SH(32) coding sequence (20). Two restriction sites, Nco I and XmaI, were introduced either side of the linker region between SH3 to the SH2 domains using polymerase chain reaction (PCR) mutagenenesis. After, treatment with Nco I and Xma I and alkaline phosphatase, a double-stranded 5' phosphorylated DNA cassette (comprising synthetic oligonucleotides 5'-CCG GTC ATC GAA GGT CGT TGC CTG GAG AAA CAT TCC TGG TAT-3' (SEQ ID NO: 7) and 5'-C ATG ATA CCA GGA ATG TTT CTC CAG GCA ACG ACC TTC GAT GA-3' (SEQ ID NO: 8)) was inserted into the pGEX2T plasmid, This oligonucleotide creates an insertion of a Factor Xa cleavage site and a $Ser^{121}Cys$ point mutation in the coding sequence. DNA sequencing, was used to confirm the presence of the insertion and mutation. The GST-Abl-SH2-TEGRC-SH2 fusion protein was expressed in E. coli DH5-α cells grown in M9 medium using ammonium chloride. Mid-log phase cells were induced with 1 mM isopropyl-1-thio-B-galactopyranoside (IPTG) for 4 hours at 37°C and harvested by centrifugation. Cells were resuspended in 4.3 mM sodium phosphate, 137 mM NaCl, 2.7 mM KCl, 1.4 mM potassium phosphate, pH 7.2, containing 100 mM EDTA, 1 mM DTT, 1 mM PMSF, 1 % v/v Triton-X and 1% w/v aprotinin and then lysed using sonication. The soluble fraction was then passed over glutathione agarose beads which were then washed with 137 mM NaCl, 8 mM sodium phosphate, 2.7 mM KCl, 1.4 mM potassium phosphate, pH 7.2. containing, 100 mM EDTA. Ab3-SH3)-IEGRC-SH2 was cleaved from the glutathione beads using thrombin (23). After thrombin cleavage, Abl-SH3-IEGRC-SH2 was exchanged to Factor Xa reaction buffer (1 mM CaCl, 100 mM NaCl and 50 mM Tris-HCl, pH 7.8, with 0.01% NaN.sub.3). About 200 units of Factor Xa (Pharmacia) were used to cleave 15 mg, Abl-SH3-IEGRC-SH2 in 4 ml reaction buffer at room temperature for 20 hours. The resulting Abl[C^{121}]SH2 was Purified by FPLC using a Superdex-75 filtration column (Pharmacia) with 137 mM NaCl, 4.3 mM sodium phosphate, 2.7 mM KCl, 1.4 mM potassium phosphate, pH 7.2, with 2 mM EDTA and 0.1 mM sodium azide as the eluent. The purified protein was concentrated to 0.5 mM using a Centricon concentrator. Purity and characterization

was confirmed by analytical HPLC and electrospray mass spectrometry: observed=11,997.8±1.4 Da, expected average isotope comp.)=11,998.2 Da.

(Page 48, line 20 through page 49, line 19)

Cloning and Expression of Abl-SH3-Intein-CBD: The gene for the Abl-SH3 domain (residues L65 to V119) was isolated by PCR from a cloned Abl-SH(32) gene (PGEX2T, (20)) using the oligonuceotide primers Abl#1 (5'-GGA TCC CCT GGT CAT ATG CTT TTT GTG GCA CTC TAT GAT TTT GTG-3')(SEQ. ID. NO.: ~~4~~ 10) and Abl#2 (5'-ATG TTT CTC CAG GCT GTT AAC GGG GGT GAT GTA GTT GCT TGG-3') (SEQ. ID. NO.: ~~5~~ 11). The PCR amplified SH3 domain was purified and digested simultaneously with Nde 1 and Hpa I and then recloned into the Ndel-Smal treated plasmid pTYB2 (New England Biolabs). The resulting plasmid, pTYB2Abl-SH3, expresses the Abl-SH3 domain fused via a single glycine residue to the intein CBD from an IPTG inducible T7 promoter. The pTYB2Abl-SH3 plasmid was shown to be free of mutations in the Abl-SH3 coding region by DNA sequencing. E. coli BL21 cells transformed with pTYB2Abl-SH3 were grown to mid-log phase in Luria Bertani (LB) medium and induced with 1 mM IPTG at 37°C. for five hours. No protein was detected by SDS-PAGE in the soluble fraction of the cell lysate under these conditions. Expression conditions were modified by inducing mid-log phase cells with 0.1 mM IPTG at room temperature for two hours to yield protein in the soluble fraction. After centrifugation, cells were re-suspended in 60 ml of lysis buffer (25 mM HEPES, pH 8.0, 0.1 mM EDTA, 250 mM NaCl, 5% glycerol, 1.0 mM PMSF) and lysed using a French press. The lysate was clarified first by low speed centrifugation and further clarified by ultracentrifugation. The clarified lysate (~45 ml) was loaded onto a 15 ml chitin column pre-equilibrated in column buffer (20 mM HEPES, pH 7.0, 250 mM NaCl, 1 mM EDTA, 0.1% Triton X-100), the column was extensively washed using the same buffer and then stored at 4°C. until further use. The column loading was determined by treating 100 .mu.l of beads overnight with a buffer containing

0.2 M phosphate, pH 7.2, 0.2 M NaCl, 100 mM dithiothreitol (DTT). Following extensive washing of the beads with 1:1 acetonitrile:water, the amount of cleaved Abl-SH3 in Solution was quantified by analytical HPLC through comparison to an Abl-SH3 standard of known concentration. This analysis indicated a loading of ~0.35 mg/ml of [G^{120}]Abl-SH3. Electrospray MS of the cleavage product: observed 6,259.4±0.5 Da, expected (average isotope COMP.)=6,260.0 Da.

(Page 49, lines 21 through 28)
Peptide Synthesis: A model peptide NH_2-CGRGRGRK[fluorescein]-$CONH_2$ (SEQ. ID. NO.: ~~3~~ 4) was chemically synthesized on an MBHA resin using in situ neutralization/HBTU activation protocols for t-butyloxycarbonyl (Boc) solid phase peptide synthesis (38). Orthogonal protection of the E-amino group of the C-terminal Lys residue with fluorenylmethoxycarbonyl allowed solid-phase attachment of fluorescein (activated as it succinimide ester) prior to the final cleavage step. The peptide was purified by reverse phase HPLC and characterized by electrospray MS: observed mass=1,245.9 0.5 Da, expected (average isotope comp.)=1,246.5 Da

(Page 51, line 29 through page 52, line7)
The tyrosine phosphorylated and unphosphorylated forms of the peptide NH_2-Cys-Glu-Asp-Asn-Glu-Tyr-Thr-Ala-Arg-Glu-aminocaproate-Lys-ϵ-[fluorescein]-CO_2H (SEQ ID NO: 2) is then designed and synthesized as described above in Example 1. This sequence is derived from the highly conserved activating autophosphorylation site of Src family kinases (9, 10). In its tyrosine phosphorylated form, a similar sequence has been shown to bind specifically to the SH2 domain of Csk (21-23). The N-terminal cysteine residue is included in the peptide to facilitate native chemical ligation. Incorporation of a carboxy-terminal fluorescent tag via a flexible linker serves as a sensitive marker of successful ligation and as a probe for further biochemical studies, including diagnostic

screening as described in Example 5 below.